SPATIAL AND DEVELOPMENTAL REGULATION OF ADIPONECTIN AND ADIPONECTIN RECEPTOR EXPRESSION IN FETAL SHEEP

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Girija Vasant Gholkar

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ABSTRACT

In human adults, adiponectin is the most abundant protein hormone found in plasma. During adulthood, adiponectin mRNA is expressed exclusively in adipose tissue and its plasma concentration is inversely related with body fat mass and insulin sensitivity. Adiponectin plays a variety of roles, including stimulation of vasodilation, oxidation of triglycerides in muscle, and suppression of glucose production in liver. Far less is known about adiponectin during fetal life, but fetal expression in non-adipose tissues of mesodermal and ectodermal origin was reported recently. The major objective of the present study was to investigate the mRNA expression of adiponectin and its receptors by qRT-PCR in fetal sheep, a recognized model of human fetal life. Adiponectin mRNA levels were found at day 135 of fetal life in perirenal adipose tissue and this expression was 2-fold higher than in adult ewes.

No significant mRNA expression was found at day 50 in muscle or other tissues surveyed (heart, intestine, liver, fetal membrane, brain, kidney, placenta, and lung). Adiponectin Receptor-1 (AdipoR1), whose mRNA is most abundantly found in skeletal muscle in adult humans, was most abundant in placenta, liver and muscle. Its mRNA levels declined by 50% in liver from day 50 to day 135, but remained unchanged in placenta and muscle. AdipoR1 expression increased 2-fold in muscle from day 135 to adult. Adiponectin Receptor-2 (AdipoR2), whose mRNA is most abundantly expressed in liver in adult humans, was also found in the placenta, liver, and muscle. Its expression declined by 60% in placenta from day 50 to day 135. Skeletal

muscle AdipoR2 expression increased 3-fold from day 135 to adult. Intrauterine Growth Retarded (IUGR) or low birth-weight fetuses did not exhibit a significantly different expression of Adiponectin, AdipoR1, and AdipoR2 from normal-sized fetuses.

BIOGRAPHICAL SKETCH

Girija Vasant Gholkar was born in the borough of Queens, New York City to Rekha and Vasant Gholkar. She has an older sister, Radha, a clinical psychologist. At age five, she moved with her family to the suburbs of New Jersey, where she grew up. She graduated with a degree in biological sciences from Rutgers University, *cum laude*. After working in the flavor and fragrance industry and the state government in a research-oriented setting, she decided to attend graduate school at Cornell University.

She loves cooking Indian and Thai food, baking pastries, community-based volunteer work, fashion, planning events, and is an avid enthusiast of Indian art. The road of life is open for her; upon completion of her M.S. degree, she plans to use the analytical and problem-solving skills she acquired in graduate school in a management consulting role within a life sciences division of a strategic consulting company. She also plans to pursue a MBA in the near future. She hopes to eventually settle in the New York City/New Jersey area.

For my parents, Rekha and Vasant, and my sister, Radha

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CHAPTER 1

INTRODUCTION

Adiponectin is a protein hormone consisting of 244 amino acids that is secreted exclusively by adipocytes in postnatal humans and rodents (Pajvani et al. 2003). Adiponectin is known to increase insulin sensitivity by decreasing glucose output in the liver and by promoting oxidation of triglycerides in skeletal muscle (Pajvani et al. 2003). Although the hormone exerts its effects predominantly in liver and muscle, it also functions in reducing inflammation in the cardiovascular system (Pajvani et al. 2003).

The protein hormone circulates in three different isoform forms in plasma: Low-Molecular-Weight (LMW), Medium-Molecular-Weight (MMW), and High-Molecular-Weight (HMW) (Pajvani et al. 2003). Female humans and mice have higher levels of circulating adiponectin, and also a higher proportion of adiponectin circulating as the HMW form (Pajvani et al. 2003). Thiazolidinediones (TZDs) which activate the nuclear receptor PPAR-γ, induce adiponectin mRNA abundance (Pajvani et al. 2003). This suggests that TZDs alleviate insulin resistance in-part by increasing plasma adiponectin (Pajvani et al. 2003).

Little is known about adiponectin mRNA and protein expression in fetal life. One study has shown that adiponectin mRNA and protein becomes apparent in human fetal life by 14 weeks of gestation (Corbetta et al. 2005). The study shows that a variety of non-adipose tissues express adiponectin, including skeletal and smooth muscle tissues (Corbetta et al. 2005). The fetal sheep has been used extensively as a model of human fetal life, but little information is available on adiponectin during sheep fetal life.

Therefore, the first objective of this study was to determine if adiponectin was expressed in fetal sheep. After detecting adiponectin mRNA levels in fetal adipose tissue, we surveyed other fetal tissues. We also assessed mRNA levels of the two adiponectin receptors in muscle and liver in early and late fetal life. Finally, we examined whether fetal growth retardation affects the adiponectin system during uterine life.

CHAPTER 2

LITERATURE REVIEW

Introduction

Obesity is associated with various pathologies, including Type 2 diabetes, dyslipidemia, hypertension, and atherosclerotic cardiovascular disease (Bobbert 2005). Formerly, adipose tissue was considered to be an inert fat-storage tissue, but in recent years, it has become clear that adipose tissue is actively involved in homeostasis and other physiological functions (Bobbert 2005). Adipose tissue is known to secrete a variety of adipocytokines which are closely involved with insulin resistance and cardiovascular disease (Bobbert 2005). Altered production of these adipocytokines may lead to pathologies related to the dreaded metabolic syndrome (Bobbert 2005). Subsequent normalization of these hormone levels may prove effective in reversing the detrimental effects brought about by the metabolic syndrome (Bobbert 2005).

Adiponectin

Structure

Adiponectin mRNA is the most abundant transcript in adipose tissue (Bobbert 2005). Adiponectin is a protein hormone which accounts for approximately 0.01% of total plasma protein, or 3-30 µg/ml in humans (Hopkins 2006). The protein hormone belongs to the soluble collagen super-family and shares structural homology with collagen VIII and X, complement factor C1q and the tumor necrosis factor family (Bobbert 2005). Human adiponectin mRNA is translated into a 247 amino acid protein, and secreted in

plasma as a 226 amino acid protein after the removal of the 21-amino acid signal peptide.

The adiponectin protein consists of four distinct regions. The first is a short signal sequence that targets the hormone for secretion outside of the cell. The second is a short hyper variable region that varies among species. The third is a 65 amino acid sequence with similarity to collagenous proteins. The last region is a globular domain which has the ability to bind three calcium ions to its head (Schraw 2008).

The human adiponectin gene is a single copy gene which is located on chromosome 3q27 (Das 2000). The gene spans 17 kb, and its cDNA is made of 3 exons and 2 introns (Das 2000). The first exon contains 30 bases, and lies 8.5 kb upstream of Exon 2 (Das 2000). Exon 2 extends over 229 bases, and contains the starting methionine codon, and encodes approximately half of the collagenous domain (Das 2000). Exon 3, which contains 4277 bases, codes for the second half of the collagenous domain (Das 2000). The substitution of T to G at nucleotide 45 (codon 15) in exon 2 and of G to T at nucleotide 276 (codon 92) in intron 2 have been associated with type 2 diabetes and obesity (Gonzalez-Sanchez 2005). Likewise, a commonly identified missense mutation in Exon 3 (R112C) is also known to be responsible for low plasma concentration (Takashi 2000), possibly leading to subsequent pathologies including Diabetes type 2 and/or obesity.

Adiponectin is modified extensively after its secretion from adipocytes in a series of post-translational modifications, or PTMs (Richards 2006). Two primary modifications it undergoes are glycosylation and hydroxylation. After analysis of endogenous adiponectin in two-dimensional gel electrophoresis, it is clear that glycosylation results in a variety of variants that differ substantially

in pI value (the pH value at which a protein has no electrical charge) and molecular mass (Richards 2006). From adiponectin isolated from bovine and mouse adipocytes, it is apparent that four conserved proline residues in the collagenous domain undergo hydroxylation, and five conserved lysine residues (one in the hypervariable region and four in the collagenous domain) are hydroxylated and subsequently glycosylated by a glucosyl-alpha(1-2) galactosyl group, as determined by NMR analysis (Richards 2006). Human adiponectin has three additional hydroxylation sites: Pro^{71}, Pro^{76}, and Pro^{95} relative to human and mouse adiponectin (Richards 2006).

Molecular forms

Adiponectin has never been found to circulate as a monomer. Instead, it circulates as three different oligomers, namely low molecular weight trimer (LMW), medium molecular weight hexamer (MMW), and a high molecular weight form containing more than 18 monomers (HMW). Each oligomer has distinct functional properties (Bobbert 2005). In addition, there is evidence that yet another primer, which is complexed to albumin, is present in the plasma (Hada 2007). It is not surprising that adiponectin would bind to albumin since the latter serves as a carrier for many molecules of low water solubility.

LMW adiponectin is known as the 'basic building block' for the other circulating adiponectin forms (Wang 2008). The trimer is formed via hydrophobic interactions within its globular heads and stabilized by the non-covalent interactions of the collagenous domains in a triple-helix stalk (Wang 2008). The medium-molecular-weight (MMW) form results from disulfide bond-mediated self-association of 2 homotrimers. The cysteine residue located in the N-terminal variable region (Cys^{36} in the human) is essential for disulfide bond formation (Tsao 2003). Under electron microscopy, MMW

adiponectin was shown to have 2 trimers adjacent to one another, in parallel head-to-head fashion (Wang 2008). In addition, replacement of Cys^{36} with either serine or alanine abolishes the formation of MMW and HMW adiponectin.

The size of HMW adiponectin varies across species. HMW adiponectin is exclusively octadecameric in mouse and cattle but is made up of 18-30 monomers in humans. The structure of octadecameric HMW adiponectin has been studied by dynamic light scattering and electron microscopy. Octadecameric HMW adiponectin has an asymmetric bouquet-like structure containing six globular objects, each corresponding to a trimer (Wang 2008). The stalks bunch together, which allows for N-terminal disulfide bonding (Wang 2008). When HMW adiponectin is viewed from the side, it appears to be a conical structure, with the C-terminal forming the base (Wang 2008).

It has been shown that adiponectin complexes do not interconvert *in vivo* (Schraw 2008). When recombinant adiponectin labeled with a fluorescent infrared dye was injected into mice, and the fluorescent hormone was subsequently analyzed by gel filtration at set time points after injection, the percent content of each complex was maintained over time (Schraw 2008).

Glycosylation and hydroxylation appear to contribute to the formation and stability of the HMW adiponectin complex (Wang 2008). The first line supporting this claim is that the HMW adiponectin complex has a much higher content of carbohydrates than the LMW and MMW adiponectin (Peake 2007). The higher level of glycosylation in HMW adiponectin is primarily attributed to an increased level of a glucosylgalactosyl moiety, rather than sialic acid (Peake 2007). Quantification of sugars on affinity-purified adiponectin from nine human plasmas showed that dimers of HMW isoforms contain a 1-3 fold-

greater amount of total sugar than LMW isoforms (Peake 2007). However, both forms contain similar amounts of sialic acid (Peake 2007). This implies that HMW adiponectin has far more glucosyl-galactosyl moieties than sialic acid residues. Furthermore, destruction of sialic acid has no effect on the stability or conformation of adiponectin, while modification of glucosyl-galactosyl moiety leads to changes in the stability of HMW and MMW complexes (Peake 2007).

Secondly, ablation of lysine hydroxylation and glycosylation by either pharmacological inhibition or mutagenesis substantially impaired the intracellular assembly and secretion of the HMW complex *in vitro* and *in vivo* (Wang 2008). In adiponectin-knockout mice infused with adenovirus expressing a mutant adiponectin in which four lysine residues are replaced with arginine, the HMW adiponectin complex was virtually undetectable in plasma, suggesting that it never formed (Wang 2008). This suggests that the glucosylgalactosyl moiety attached on these lysine residues facilitates formation of HMW adiponectin (Wang 2008).

It has been shown that the various adiponectin complexes are relatively stable under normal conditions (Schraw 2008). A human serum sample containing various adiponectin oligomers was subjected to two freeze-thaw cycles (Schraw 2008). A single freeze-thaw cycle did not alter the proportion of the various adiponectin complexes, and only slight changes occurred after the second cycle (Schraw 2008). This shows that standard storage methods will not affect the integrity of the various adiponectin complexes (Schraw 2008).

Other conditions, however, have an effect on the integrity of adiponectin. In particular, pH is a factor that significantly affects adiponectin

stability (Schraw 2008). All adiponectin isoforms are relatively stable at basic pH, but acidic conditions leads to the disaggregation of HMW adiponectin (Schraw 2008). A small change in pH from 7 to 6 results in the transformation of HMW to the LMW adiponectin. At pH 4, adiponectin may break down further into monomers or precipitate (Schraw 2008).

Calcium also affects the stability of human adiponectin. In the absence of calcium, human adiponectin is not soluble and precipitates on the column during purification (Schraw 2008). When a calcium-binding mutant strain of the hormone is created and purified in a buffer containing calcium, the mutant is not affected by changes in calcium, therefore implying that this mutant is insensitive to changes in calcium concentration (Schraw 2008).

Adiponectin Secretion

A high proportion of adiponectin is retained within the adipocytes, and treatment of cells with reducing agents such as 2-mercaptoethanol stimulates its secretion (Wang 2008). This implies that adiponectin is trapped in the adipocyte by a thiol-mediated retention, possibly caused by a covalent interaction between adiponectin and an endoplasmic reticulum (ER)-resident protein (Zhao 2007). Since adiponectin requires extensive post-translational modifications to mature, thiol-mediated retention may prolong its residence within the secretory pathway, thereby facilitating proper folding into the HMW complexes (Anelli 2003).

Secretion of all the adiponectin oligomers from adipoctyes is regulated by a pair of molecular chaperones in the endoplasmic reticulum (Zhao 2007). These two entities, ERp44 (an ER protein of 44 kDa) and Ero1-Lα are both induced during adipogenesis (Zhao 2007). ERp44 is an ER protein that binds adiponectin. Knockdown of ERp44 expression by *si*RNA increases

adiponectin secretion, consistent with this protein being involved in intracellular adiponectin retention. This does not occur for the adiponectin mutant in which cys39 is substituted for serine.

In contrast, over-expression of Ero1-Lα selectively enhances the secretion of HMW adiponectin (Wang 2008) whereas siRNA-mediated down-regulation of Ero1-Lα inhibits the secretion of HMW adiponectin, but enhances the release of trimeric adiponectin (Qiang 2007). Therefore, we can conclude that Ero1-Lα is a stimulator of HMW adiponectin release from adipocytes, and that ERp44 and Ero1-Lα have opposing effects on the control of the maturation of secretion of adiponectin oligomers (Qiang 2007).

Ero1-Lα is causally involved in mediating the effect of other factors that regulate adiponectin secretion. PPAR-γ agonists, such as thiazolidinediones (TZDs) selectively increase the secretion of HMW adiponectin by increasing the expression of Ero1-Lα (Wang 2008). Conversely, SIRT-1, a NAD-dependent deacetylase, inhibits the secretion of HMW adiponectin (Qiang 2007). SIRT-1 deacetylates PPAR-γ into its inactive form, which results in down-regulation of the PPAR-γ responsive Ero1-Lα gene (Wang 2008). This down-regulation leads to a substantial decrease in the secretion of HMW adiponectin (Wang 2008). Glucose and lactate, on the other hand, inhibit SIRT-1 (Qiang 2007). This inhibition causes activation of PPAR-γ and therefore an up-regulation in Ero1-Lα, thereby increasing the secretion of the HMW adiponectin oligomer (Wang 2008).

Analysis of Various Adiponectin Forms

There are several methods that have been developed over the last five years to separate the different oligomeric forms of adiponectin. The first method involved velocity sedimentation followed by Western blot analysis.

Though this method gained short-lived popularity, it cannot differentiate the LMW and MMW complexes of adiponectin (Pajvani 2003). SDS-PAGE under non-heating, non-reducing conditions followed by Western blot analysis, is a less time-consuming method, and allows for separation and semi-quantification of the different adiponectin oligomers (Waki 2003). This technique is regularly employed by many research laboratories (Wang 2008). Gel filtration followed by accurate quantification of adiponectin using sandwich ELISA is the most reliable, broadly available method for measurement of the various adiponectin oligomeric complexes (Kobayashi 2004).

More recently, two reports have described immune-based methods that bypass the need for size separation. Ebinuma and colleagues developed an ELISA that allows for the sequential measurement of different adiponectin oligomers in a single assay (Ebinuma 2008). In this method, two proteases are used in parallel tubes to selectively digest the LMW form or both the LMW and MMW forms (Ebinuma 2008). Total adiponectin is measured in the absence of proteases. LMW and MMW levels are obtained by differences (Ebinuma 2006).

On the other hand, Nakano and colleagues developed a monoclonal antibody that recognizes only HMW adiponectin (Nakano 2006). Not only does this method effectively evade detection of trimeric and hexameric isoforms, it also allows for the quantification of HMW adiponectin in only a few hours (Nakano 2006).

Regulation of Adiponectin mRNA Abundance

Spatial and Developmental Regulation

Northern blot analysis first confirmed the presence of adiponectin mRNA in human adipocytes (Scherer 1995). The hormone is expressed exclusively in adipose tissue in most adult animals (Kadowaki 2006). The protein is also induced 100-fold during adipocyte differentiation (Scherer 1995). This period also coincides with induction of the mRNA for other adipocyte-specific proteins such as GLUT-4 (Scherer 1995). This result was confirmed by Western blot analysis (Scherer 1995). Unlike humans and most mammals, adiponectin mRNA abundance in chickens is not limited to the adipose tissue (Maddineni 2005). By RT-PCR and Northern analysis, chicken adiponectin mRNA transcripts were also detected in liver, anterior pituitary, diencephalon, kidney, and skeletal muscle (Maddineni 2005). Abundance in these non-adipose tissues was highest in the liver (27% of adipose tissue mRNA abundance) and lowest in skeletal muscle (less than 1% of adipose tissue).

During human fetal life, adiponectin is expressed in tissues of mesodermic origin in mid-gestation, namely white and brown adipocytes, smooth muscle cells, skeletal muscle fibers of diaphragm and iliopsoas, smooth muscle cells of the small intestine and arterial walls, the perineurium and renal capsule, as well as a few select tissues of ectodermic origin (i.e. the epidermis and ocular lens) (Corbetta 2005). Interestingly, the protein hormone is not found in the placenta. In addition, protein expression of the hormone in non-adipose tissue declines with advancing gestation (Corbetta 2005). Cord blood adiponectin levels positively correlate with gestational age at delivery,

whereas they do not show any correlation with fetal birth weight or corresponding maternal levels (Corbetta 2005).

Sivan (2003) speculated that fetal circulation contains high levels of adiponectin because adipocytes are small and do not yet express signals that suppress adiponectin production (Sivan 2003). This factor may explain the high levels of adiponectin in cord blood despite a limited amount of fat in neonates (Sivan 2003). The high proportion of brown adipose tissue may also explain the phenomenon of high cord blood adiponectin.

Interestingly, no difference was found in adiponectin levels between male and female neonates (Sivan 2003). Also, no correlation was found between cord and maternal adiponectin concentration, maternal body mass index, cord leptin, or insulin levels (Sivan 2003). In addition, cord adiponectin levels are significantly higher than maternal levels at birth. Plasma adiponectin levels in the fetus do not fall upon birth (Sivan 2003). This suggests that adiponectin present in the fetal circulation is not from the placenta or from the maternal circulation (Sivan 2003). Another observation that would substantiate this claim is the fact that pregnancy induces an insulin-resistant state in the mother, while initiating the opposite effect in the fetus (Basu 2008). Since high adiponectin levels correlate with increased insulin-sensitivity, this observation would fit in with this model.

Sexual Dimorphism

When the various isoforms of adiponectin are analyzed by gel filtration chromatography and velocity sedimentation, female mice have a significantly higher level of total adiponectin in blood serum than do their male counterparts. In particular, females have a higher percentage of HMW adiponectin (approximately half of total adiponectin), with the remainder being

an even split between the MMW and LMW forms (Schraw 2007). By contrast, males have an equal distribution of plasma adiponectin among HMW, MMW, and LMW forms (Schraw 2007). Furthermore, there is no substantial gender difference in concentration between the two lower molecular weight isoforms (Xu 2005).

In both human and mice adipocytes, the ratio of HMW to total adiponectin is similar in males and females, yet the same ratio is significantly lower in the male circulation (Xu 2005). Thus, the lower fraction of adiponectin as HMW has been attributed to the fact that males have high levels of testosterone, which inhibit the secretion of this oligomer from adipocytes (Xu 2005). Castration of mice results in elevated levels of HMW adiponectin in circulation, comparable to that of female mice (Xu 2005). Ovariectomy, on the other hand, does not have any effect on the circulating level of adiponectin (Xu 2005).

A number of clinical observations support this theory. For example, after hypogonadal patients with testicular failure undergo testosterone replacement therapy, there is a striking decrease in HMW circulating adiponectin, yet only a slight decrease in total adiponectin. A recent study in Danish schoolchildren demonstrated that circulating amounts of the HMW complex were substantially less in pubertal boys than pubertal girls (Andersen 2007). The same study also showed that the ratio of HMW to total adiponectin also decreased when comparing pre-pubertal and post-pubertal males, respectively (Andersen 2007).

It is important to note that neither castration nor testosterone treatment affects the abundance of mRNA for adiponectin gene in adipocytes (Wang 2008). This suggests that testosterone selectively inhibits the secretion of

HMW adiponectin from adipocytes, without affecting the other two oligomers (Wang 2008).

Nutrition and Obesity

Despite being produced in adipose tissue, plasma adiponectin levels are inversely correlated with obesity. Plasma adiponectin concentrations and mRNA abundance were decreased in obese humans, despite increased adiposity, and also in patients that exhibited insulin resistance (Maeda 2001). When adiponectin expression was measured in subjects covering a wide range of BMI and percentage body fat, there was indeed a significant inverse association between BMI and plasma adiponectin (Kern 2003).

Fasting of obese and normal weight human subjects for a 72-hour period did not alter adiponectin levels when plasma samples were analyzed at 4-hour intervals (Merl 2005). These results imply that plasma adiponectin levels are not subject to acute nutritional influences in either obese or lean human subjects (Merl 2005).

Overfeeding either animal or human subjects is another way to observe changes in plasma adiponectin concentration. In pregnant sheep, feeding excess energy maintenance requirements increases the mRNA abundance of genes encoding lipogenic enzymes. In particular, as demonstrated by RT-PCR, mRNA levels of PPAR-γ, a master transcriptional regulator of lipogenesis increases in overfed ewes as compared with controls, leading to a subsequent increase in dependent genes such as adiponectin (Muhlhausler 2006).

Metabolic Hormones

PPAR-γ and its synthetic ligand counterparts, thiazolidinediones (TZDs), increased mRNA for adiponectin in the adipose tissue of humans

(Maeda 2001). In addition, these effects took place through transcriptional activation of the adiponectin gene (Maeda 2001). PPAR-γ agonists strongly induced adiponectin mRNA abundance in cultured 3T3-L1 preadipocytes (Combs 2002). Therefore, mechanisms underlying positive effects of PPAR-γ on circulating adiponectin may include its ability to promote adipogenesis, as well as its ability to stimulate adiponectin gene transcription (Combs 2002).

Interestingly, PPAR-γ is known to selectively increase circulating levels of HMW adiponectin. Diabetic patients and animal models that have undergone treatments with TZDs experience preferential increase in circulating levels of HMW adiponectin (Wang 2008). Furthermore, treatment of human adipocytes with TZD dose-dependently increased the secretion of the HMW isoform into the extracellular medium with no significant effect on adiponectin mRNA levels and secretion of other isoforms (Wang 2008). Similar effects have been observed in mouse adipocytes (Wang 2008).

Tumor-necrosis-factor-α (TNF-α), a cytokine involved in inflammation, on the other hand, reduces the abundance of mRNA for adiponectin (Maeda 2001). Interestingly, TNF-α is secreted during the insulin-resistant state, increasing the likelihood of obesity (Maeda 2001). However, this negative effect of TNF-α can be reversed by TZDs (Maeda 2001).

Insulin is known to be a negative regulator of adiponectin production (Wang 2008). An inverse correlation between serum levels of adiponectin and insulin is apparent when analyzing Type 1 Diabetic patients with insulin deficiency. These subjects have higher serum levels of adiponectin than their healthy control counterparts (Trujillo 2005). Furthermore, adipose-specific deletion of insulin receptor in mice also resulted in a 60% increase in circulating levels of adiponectin when compared with wild type controls

(Nawrocki 2006). Serum levels of total adiponectin also markedly increase in patients with a genetically defective insulin receptor, or with an acquired loss of an insulin receptor (Semple2006). It is important to note that this insulin-mediated reduction in serum levels of total adiponectin which is observed in mice and humans is primarily due to a preferential decrease of the HMW oligomeric adiponectin, rather than the other two isoforms (Wang 2008). Repeated studies in mice have shown that an acute injection of insulin resulted in a selective reduction of HMW oligomeric adiponectin without a significant effect on the other two forms (Wang 2008).

Metabolic Actions of Adiponectin are Mediated through AdipoR1 and AdipoR2

AdipoR1 and AdipoR2 receptors have seven transmembrane domains with an internal N-terminus and an external C-terminus (Niemann 2008). There is low sequence homology between AdipoR1 and AdipoR2 mRNA nucleotide sequences. Both receptors have low sequence homology to G-protein-coupled receptors. AdipoR1 and AdipoR2 are highly conserved across species including chickens (Ocon-Grove 2008), but have little homology to each other. In humans, AdipoR1 gene is located on chromosome 1 (q32.1) and encodes a 375 amino acid protein (Crimmins 2007). AdipoR1 mRNA is most abundant in skeletal muscle in humans and mice, though it is also expressed in human adipose tissue and pancreatic β-cells in both of these species (Zhang 2005). In chickens, the high levels of AdipoR1 mRNA are seen not only in skeletal muscle, but also in adipose tissue and the diencephalon, followed by kidney, ovary, liver, anterior pituitary gland, and spleen (Ramachandran 2006). AdipoR1 signals through through the AMPK

pathway (Crimmins 2007). AdipoR1 has extremely high affinity for the globular domain of adiponectin.

The human AdipoR2 gene is located on chromosome 1 and consists of 97K base pairs and encodes 37 amino acids (Crimmins 2007). AdipoR2 mRNA is most abundant in liver in humans and mice, with lower levels in skeletal muscle (Zhang 2005). It, like AdipoR1, also shows mRNA abundance in both human and mouse adipose tissue and pancreatic β-cells (Zhang 2005). In the chicken, however, AdipoR2 mRNA abundance is highest in adipose tissue, followed by skeletal muscle, liver, ovary, diencephalon, anterior pituitary gland, kidney, and spleen (Ramachandran 2006). AdipoR2 signals through activation of PPAR-α. AdipoR2 has lesser affinity for adiponectin globular domain than AdipoR1.

The functions of both AdipoR1 and AdipoR2 are mainly exerted in the muscle and liver, respectively, in adult humans (Zhang 2005). Both receptors mediate the decrease of fatty acid buildup in the muscle and liver by up-regulating enzymes involved in fatty acid oxidation (Menzaghi et a. 2007). In the muscle, adiponectin-AdipoR1 binding stimulates the translocation of GLUT4 receptors to the periphery of the muscle, thereby stimulating the uptake of glucose into the muscle (Menzaghi et al. 2007). Likewise, in the liver, adiponectin-AdipoR2 binding decreases the output of glucose from the organ by down-regulating hormones involved in gluconeogenesis (Menzaghi et al. 2007).

Diabetes and obesity-related pathologies cause a reduction in levels of mRNA for these receptors (Niemann 2008). Some studies suggest that mRNA levels of both AdipoR1 and AdipoR2 are significantly decreased in muscle and adipose tissue of insulin-resistant *ob/ob* mice, which exhibit hyperglycemia and

hyperinsulinemia as compared with control mice (Kadowaki 2005). Moreover, adiponectin-induced activation of AMPK is impaired in skeletal muscle of *ob/ob* mice (Kadowaki 2005). These data suggest that adiponectin resistance is observed in *ob/ob* mice. Induction of diabetes with streptozotocin caused an increase in AdipoR1 and AdipoR2 mRNA abundance in mouse skeletal muscle (Kadowaki 2005). A similar increase was seen after fasting in humans (Kadowaki 2005). These data suggest that insulin negatively regulates AdipoR1 and AdipoR2 expression.

However, in a study involving Zucker Diabetic rats (ZDF), this model was compared with control rats (L) at three time periods; 7 weeks (when the ZDF rats were insulin resistant), at 14 weeks (ZDF had diabetes mellitus), and 21 weeks (ZDF had both diabetes and insulin deficiency) (Metais et al. 2008). During these three time periods, the ZDF received no treatment, or either fenofibrate or metformin, two PPAR-α agonists (Metais et al. 2008). The L group did not receive treatment at any time (Metais et al. 2008). In the ZDF group, as compared to the L group, both AdipoR1 and AdipoR2 mRNA levels decreased in white adipose tissue (WAT) with increasing age, yet increased in liver. No change in mRNA levels was noted in muscle (Metais et al 2008). In addition, fenofibrate was responsible for a moderate increase in both receptors' mRNA levels in liver, while metformin increased abundance of mRNA for both receptors in muscle and for AdipoR1 in WAT. This may imply that neither adiponectin receptor plays an important role in the development of insulin resistance and diabetes (Metais et al. 2008).

Adiponectin has more recently been shown to bind T-cadherins (Hug 2004). Cadherins comprise comprise a large family of cell-surface proteins involved in calcium-mediated cell-cell interactions and signaling (Hug 2004).

T-cadherin has more of an affinity for the MMW and HMW adiponectin multimers (Whitehead 2005). In addition, only eukaryotically expressed adiponectin binds to T-cadherin, implying that post-translational modifications of the protein hormone are necessary for binding (Hug et al. 2004). Adiponectin mutants lacking a conserved N-terminal cysteine residue necessary for formation of both the MMW and HMW form did not effectively bind to T-cadherin (Hug et al. 2004). Finally, adiponectin is expressed throughout the human body, but at highest levels in smooth muscle and endothelium of the vascular system, with a lesser level of mRNA in the muscle (Hug et al. 2004). Since adiponectin plays a role in protection from coronary artery disease and other cardiovascular pathologies by decreasing inflammation, cellular adhesion, and foam cell production, this suggests that T-cadherin may be involved in the beneficial effects of adiponectin in the vasculature (Hug et al. 2004, Menzaghi et al. 2007).

Function

Adiponectin has been shown to lower plasma glucose levels in mice, irrespective of plasma insulin levels, suggesting that adiponectin is important for insulin sensitivity (Hopkins 2006). When wild type C57B1/6J mice were given a single injection of adiponectin into the peritoneal cavity, a significant decrease in circulating glucose occurred (Berg 2001). In addition, when a similar injection of the hormone was given to *ob/ob* mice, they displayed a similar decrease in serum glucose levels, but not an increase in insulin levels (Berg 2001). This fuels the hypothesis that adiponectin sensitizes the liver and muscle to the effects of circulating insulin, resulting in a decrease in hepatic glucose output (Berg 2001).

Similar studies in mice have shown a 65% reduction in the rate of glucose production after the simultaneous infusion of adiponectin and a pancreatic euglycemic clamp, which serves to maintain a constant glucose concentration in the plasma (Combs 2001). Glucose flux through Glucose-6-phosphotase was also shown to decrease with adiponectin administration, whereas the activity of the direct pathway of glucose 6-phosphate biosynthesis (and index of hepatic glucose phosphorylation) increased significantly (Combs 2001). This shows that an increase in circulating levels of adiponectin inhibits both the expression of hepatic gluconeogenic enzymes and the rate of endogenous glucose production (Combs 2001).

Numerous loss/gain of function studies have shown that adiponectin plays a protective role against insulin resistance *in vivo.* Insulin sensitivity is reduced in mice when adiponectin is reduced by genetic factors, (i.e. a single nucleotide polymorphism of the adiponectin gene), or by environmental factors (i.e. a high fat diet) (Kubota 2002). Mice heterozygous for a null adiponectin allele have a 30-60% deficiency in circulating plasma adiponectin levels and are mildly insulin resistant (Kubota 2002). However, body weight is not affected (Kubota 2002). This suggests that despite adiponectin's leading role in insulin sensitivity, the hormone may not have as important a role in appetite control as previously thought (Kubota 2002). Similar results have been found in *in vitro* studies. In adipocytes, adiponectin overexpression increases the ability of insulin to maximally stimulate glucose uptake through GLUT4 gene expression (Fu 2005). Adiponectin also increased GLUT4 recruitment to the plasma membrane (Fu 2005). This evidence further validates the role that adiponectin plays in insulin sensitivity.

HMW adiponectin appears to be the form most important in promoting insulin action. Recombinant adiponectin produced from mammalian cells, which can form HMW oligomers, decreased hyperglycemia in diabetic mice through inhibition of hepatic glucose production (Berg 2001). In contrast, bacterially-generated full length adiponectin, which lacks the ability to form HMW adiponectin, was ineffective at reducing blood glucose levels (Berg 2001). The sheer fact that bacteria are not able to introduce post-translational modifications to adiponectin not only suggests that HMW adiponectin is likely to be the biologically active oligomer, but also implies that modifications are necessary for creation of the HMW multimers (Berg 2001).

Other studies have extended these observations by investigating the relation between oligomer distribution and various health states (Wang 2008). For example, Pajvani et al. have found that an increase in HMW adiponectin rather than total adiponectin is associated with an increase in insulin sensitivity (Pajvani et al. 2003). The fact that the diabetic mouse model, *db/db*, has a far lower percentage of circulating adiponectin in the HMW form despite similar total adiponectin levels compared with phenotypically normal heterozygote and wild type littermates lends support to this hypothesis (Pajvani et al. 2003). In human subjects with Type II Diabetes, there is a decrease in the proportion of HMW to total adiponectin, suggesting these two events are causally linked with the disease (Wang 2008).

Likewise, similar oligomer concentration differences have also been identified in other disease states. In human patients with coronary artery disease, the percentage of HMW per total adiponectin is significantly lower than in healthy control subjects, whereas the MMW form is similar, and the trimer form significantly higher (Kobayashi 2004). After a moderate weight

reduction in these subjects, however, the HMW form increases and the hexamer and trimer forms substantially decrease (Kobayashi 2004).

Endothelial dysfunction is also shown to be correlated with obesity. This is reflected by the fact that patients who suffer from coronary artery disease have a lower percentage of HMW per total adiponectin, have a similar hexamer concentration, and a higher trimer concentration, as do patients with hyperinsulinemia and Type 2 diabetes (Kobayashi 2004). Both in vitro and in vivo studies show that adiponectin exercises a protective effect on endothelial cells, most likely due to its production of nitric oxide, a potent vasodilator, thereby alleviating the pathogenic effects of obesity on vascular function (Chen et al. 2003). Adiponectin is also known to have anti-apoptotic actions in endothelial cells (Hopkins 2006). These actions are dependent on the initiation of AMPK signaling (Hopkins 2006). In addition, these anti-apoptotic effects are only observed with the HMW adiponectin form. Furthermore, since adiponectin-knockout mice exhibit impaired endothelial-dependent vasodilation, it is appropriate to infer that adiponectin plays a crucial role in maintaining vascular function and tone (Hopkins 2006).

Although the effects of the HMW oligomer dominate in terms of insulin sensitivity, the two smaller complexes have a more pronounced central effect (Wang 2008). It has been shown that the trimeric and hexameric oligomers are found in high concentrations in the cerebrospinal fluid (CSF), yet the HMW complex is virtually undetectable (Kusminski 2007). This is perhaps because the HMW complex is so large, that it cannot cross the blood-brain barrier (Kusminski 2007). Interestingly, despite the increased insulin-sensitizing effects of the protein, mice injected with the hexamer form have higher food intake and decreased energy expenditure (Wang 2008).

CHAPTER 3

MATERIALS AND METHODS

General Information

All procedures were approved by the Cornell University Institutional Animal Care and Use Committee (IACUC). Ewes (3-6 years old) were time-bred to obtain fetuses of appropriate gestational age. The number of fetuses carried by each ewe was determined at 38-60 days post-coitus by ultrasound sonography (Greenwood et al 1998). Throughout pregnancy, ewes were fed a single total mixed ration (TMR) at or above predicted nutrient requirements.

Animals and Design

Tissues at Gestational Day 50 and day 130

A group of 14 Finn X Dorset ewes was sacrificed between day 40 and day 50 post coitus (G50) and another between day 130 and 140 (G130) (Greenwood et al 1998). This group included ewes previously used to describe fetal leptin expression (Ehrhardt et al 2002). At the time of slaughter, ewes had an average body condition score of 3.6 ± 0.2 [1=thin, 5=fat]. In addition, 4 ewes were slaughtered specifically for this study (2 at day 50 post coitus and 2 at day 130 post coitus). A total of 9 ewes were slaughtered at day 50, and 9 more at day 135.

Ewes were slaughtered by captive-bolt stunning and exsanguination (Ehrhardt et al. 2002). Fetuses were promptly removed from the ewe and their associated placentomes were weighed (Ehrhardt et. al 2002). Fetal and maternal tissues were dissected and snap frozen at -80°C. Fetal tissues collected include muscle, placenta, heart, lung, liver, fetal membrane,

intestine, and brown adipose tissue (perirenal fat). Maternal tissues collected include liver, subcutaneous fat, perirenal fat, and skeletal muscle. In addition, blood was obtained from each ewe by jugular venipuncture and from fetuses by cardiac puncture. Plasma was prepared by heparin addition (20 KU/liter) and centrifugation at 3000 rpm for 15 minutes.

Effect of IUGR at G130

A group of 12 Finn X Dorset ewes was selected at the time of sonography for having 2 or more fetuses. Ewes were maintained at body condition score range of 2.5-3.5 from the time of mating until sacrifice between day 131-133 post coitus as described above. All fetuses were weighed within 5 minutes post-mortem. Fetuses were categorized as intrauterine growth retarded (IUGR), if weighing less than 2.9 kg and normal if weighing more than 3.8 kg. A total of 16 fetuses were slaughtered, 8 IUGR, and 8 normal-sized. Blood was obtained by cardiac puncture and plasma was prepared as previously described. Muscle and liver was obtained by dissection.

RNA Extraction and Reverse Transcription

Total RNA was extracted by affinity chromatography (RNeasy® Mini Kit, Qiagen Inc., Valencia, Ca). Approximately 150 mg of tissue was homogenized with a polytron in 1.5 mL of Qiazol solution (Qiagen, Valencia, CA). Total RNA was purified using RNeasy Mini columns and on-column RNase-free DNase treatment according to the manufacturer's protocol (Qiagen). The concentration of total RNA was measured by absorbance at 260 nm. Quantity and integrity of RNA were determined using the RNA Nano Lab Chip Kit (Agilent; Palo Alto, CA, USA). Reverse transcription reactions were performed

using a commercial kit exactly as described by the manufacturer (High Capacity cDNA Synthesis Kit, Applied Biosystems, Foster City, CA).

Primer Selection for Sybrgreen© Assay

At this time, full length cDNAs are not available for ovine adiponectin, AdipoR1, and AdipoR2. The following strategy was used for each gene to identify primers for the SYBR green RT-PCR assays. First, bovine adiponectin, AdipoR1, and AdipoR2 cDNAs were obtained and exon spanning boundaries were deduced by comparison with bovine genomic sequence. Second, bovine cDNAs were used as target cDNAs to obtain primer pairs using a commercial primer design software (OligoPerfect^{TM} Designer, www.tools.invitrogen.com). Finally, the bovine cDNAs were blasted against the currently available ovine genome sequence (International Sheep Genomic Consortium, www.isgcdata.agresearch.co.nz). For each primer, mismatched nucleotides were changed to the nucleotide present in the ovine exonic sequence. All oligonucleotide primers were custom made by Invitrogen.

Measurement of Gene Expression

Real time SYBR green PCR assays were performed using primers given in Table 1. Reactions were performed in triplicate in a final volume of 20 µL using ABI Power SYBR Mix (Applied Biosystems). Reactions contained 500 nM of each primer and diluted cDNA (0.5 to 5 ng for Adiponectin, 5 ng for AdipoR1 and R2 and 5 ng for 18S) and were performed in a Thermal cycler (GeneAmp PCR System 2400, Perkin Elmer Corporation, Norwalk, CT). A relative standard curve was constructed for each assay and consisted of 7-9 serial dilutions of pooled cDNAs. The pool consisted of adult subcutaneous

adipose tissue for Adiponectin, muscle tissue for AdipoR1, and liver for AdipoR2. Amplification was linear and efficient across the range of standards for each assay (efficiency for all assays was >0.98, based on efficiency = $10^{(-1/slope)}$-1, where slope is obtained from regression of C_T verses log input). Each primer pair yielded a single product based on melting curve analysis. Unknown sample expression was determined from the appropriate standard curve and expressed as a fold difference. 18S rRNA abundance was unaffected by tissue age or fetal size and was used as the invariant control.

Table 1: Nucleotide sequence of oligonucleotide primers used in Realtime® RT-PCR assays. For each transcript, the sequence of the forward primer is labeled (F), and for the reverse primer it is labeled (R). The exon location of each primer is based on bovine genome sequence.

Target Gene	**Primers**	**Exon**	**Product Length**
Adiponectin	F-GTGGCTCTGATTCCACACCT	1	83 bp
	R-GCCATGACTGGGCAGG5GCTA	2	
AdipoR1	F-CATGGAGAAGATGGAGGAGTTC	3	110 bp
	R-CGTGCAGCAGGTAGTC	4	
AdipoR2	F-CCCGGCAAGTGTGACATCT	6	109 bp
	R-TTCGAGACCCCGTGGAAGT	7	

Table 2: Models Used

Models			
Figures	Response	Predictors*	Transformation?
Figure 1	Adiponectin	Tissue, Age, Tissue x Age	No
Figure 2	Adiponectin	Tissue, Age, Tissue x Age	Square Root of % of d135 Adipose Tissue
Figure 3	Adiponectin	Tissue, Age, Tissue x Age	Square Root of Adiponectin/18s
Figure 4	Adiponectin	Size	No
Figure 5	AdipoR1	Tissue, Age, Tissue x Age	No
Figure 6	AdipoR1	Tissue, Age, Tissue x Age	No
Figure 7	AdipoR1	Size, Tissue, Size x Tissue	No
Figure 8	AdipoR2	Tissue, Age, Tissue x Age	No
Figure 9	AdipoR2	Tissue, Age, Tissue x Age	No
Figure 10	AdipoR2	Size, Tissue, Size x Tissue	No

*18s is a predictor for all model

Statistical Analysis

The data were analyzed by a general linear model procedure of JMP, a statistical software package (SAS Institute, Incorporated, Cary, NC). Specifically, an analysis of covariance was performed. Depending on the study, the response was either adiponectin mRNA abundance, AdipoR1 mRNA abundance, or AdipoR2 mRNA abundance. In all cases, the covariate was the abundance of 18s rRNA. The treatments were a subset of tissue type (muscle, kidney, liver, placenta, fetal membrane, lung, heart, brown adipose tissue, perirenal fat, subcutaneous fat, and intestine), age (d50, d135, or adult), and size (normal or IUGR), and their interactions. Individual means were compared using the Least Squares Means Differences Student's t Test (in JMP). In some of the models, the residuals of the analysis of covariance did not follow an approximately normal model. This was dealt with by transformation of the response. See Table 2 above for more details of the models used and for the corresponding figures in the results section.

CHAPTER 4

RESULTS

Adiponectin mRNA Abundance

First, we asked whether adiponectin is expressed in fetal tissue. Therefore, adiponectin mRNA abundance was measured by qRT-PCR in fetal perirenal fat at day 135, as well as adult perirenal and subcutaneous fat. Adiponectin mRNA abundance was significantly higher in fetal than Adult perirenal fat (Figure 1, Tissue $P<0.002$). Adiponectin mRNA was similar in adult perirenal and subcutaneous adipose tissue (Figure 1).

Next, we quantified adiponectin mRNA abundance in various fetal tissues at day 50 and day 135 of gestation and expressed these data as a percent of relative value in day 135 perirenal adipose tissue. Abundance of mRNA was extremely low in heart, intestine, liver, fetal membrane, brain, kidney, placenta, muscle, and lung (Figure 2). At day 135, however, mRNA in skeletal muscle rose to ~15% of adipose tissue expression (Age x Tissue, $P<0.01$).

Next, we asked whether adiponectin mRNA abundance persisted in liver, kidney, and skeletal muscle over the times considered. There is a significant Age x Tissue interaction ($P<0.001$). From Figure 3 we see that mRNA levels in the liver and kidney are negligible at all three time points, but that abundance appears to increase over time in muscle. We tested individual means by using t-tests. The p-value for the difference between day 50 and day 135 is 0.0663. Although this is larger than .05, it is very small and suggests a tendency. The comparison between day 135 and adult has a p-value of 0.0330, which suggests a significant difference. The comparison

between day 50 and adult has a p-value of 0.0006, which again suggests a significant difference. Thus the level in muscle increases in time.

Finally, adiponectin mRNA was compared in day 135 perirenal adipose tissue obtained from growth-retarded fetuses (less than 2.9 kg at birth) and normal-sized fetuses (more than 3.8 kg at birth). No significant difference in adiponectin mRNA abundance was detected (Figure 4).

AdipoR1 mRNA Abundance

Adiponectin receptor mRNA abundance is at high levels in postnatal muscle and liver. To ascertain if adiponectin receptor-1 (AdipoR1) is expressed during fetal life, we compared day 135 and adult liver and muscle (Figure 5). AdipoR1 mRNA abundance increased from fetal to adult life but only in muscle (Age x Tissue, $P<0.03$). AdipoR1 mRNA muscle levels increased roughly 2-fold from fetal day 135 to adulthood ($P<0.005$).

Next, we asked whether AdipoR1 mRNA abundance changed through fetal life (Figure 6). AdipoR1 was quantified at day 50 and day 135 of fetal life in placenta, muscle, and liver. No difference in mRNA abundance was noted between day 50 and day 135 for placenta and muscle, but liver mRNA abundance was reduced between day 50 and day 135 of fetal life (Age x Tissue, $P<0.01$). The drop of mRNA abundance was approximately 50% ($P<0.0001$).

Finally, we determined whether AdipoR1 mRNA abundance was affected by fetal size at day 135 of gestation. Although AdipoR1 mRNA abundance was numerically higher in liver and muscle of small fetuses, a significant difference in mRNA levels was not detected (Figure 7).

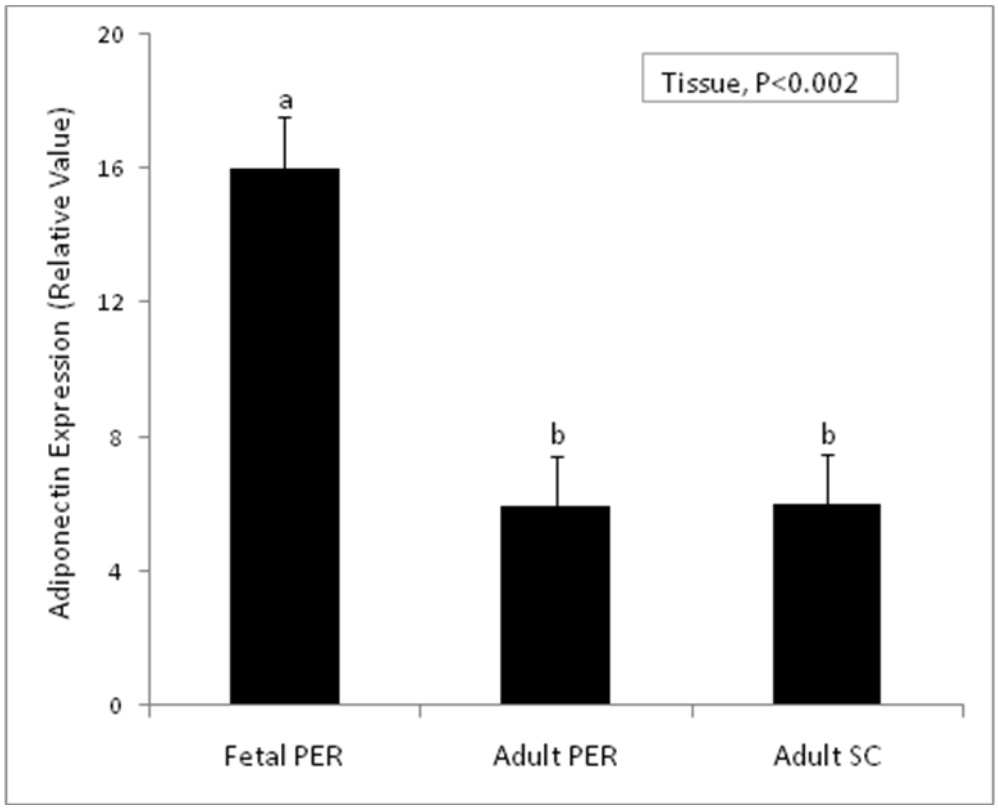

Figure 1: Effect of development on adiponectin mRNA in ovine adipose tissue. Adiponectin mRNA relative to 18s rRNA was measured in perirenal tissue obtained at day 135 of fetal life (Fetal PER) and in perirenal and subcutaneous tissue obtained from adult ewes (Adult PER and Adult SC). Adiponectin mRNA was measured by quantitative real-time RT-PCR. Each bar represents the mean ± SE of 4 animals. The significant effect of tissue is reported and bars with different letters differ at $P<0.03$.

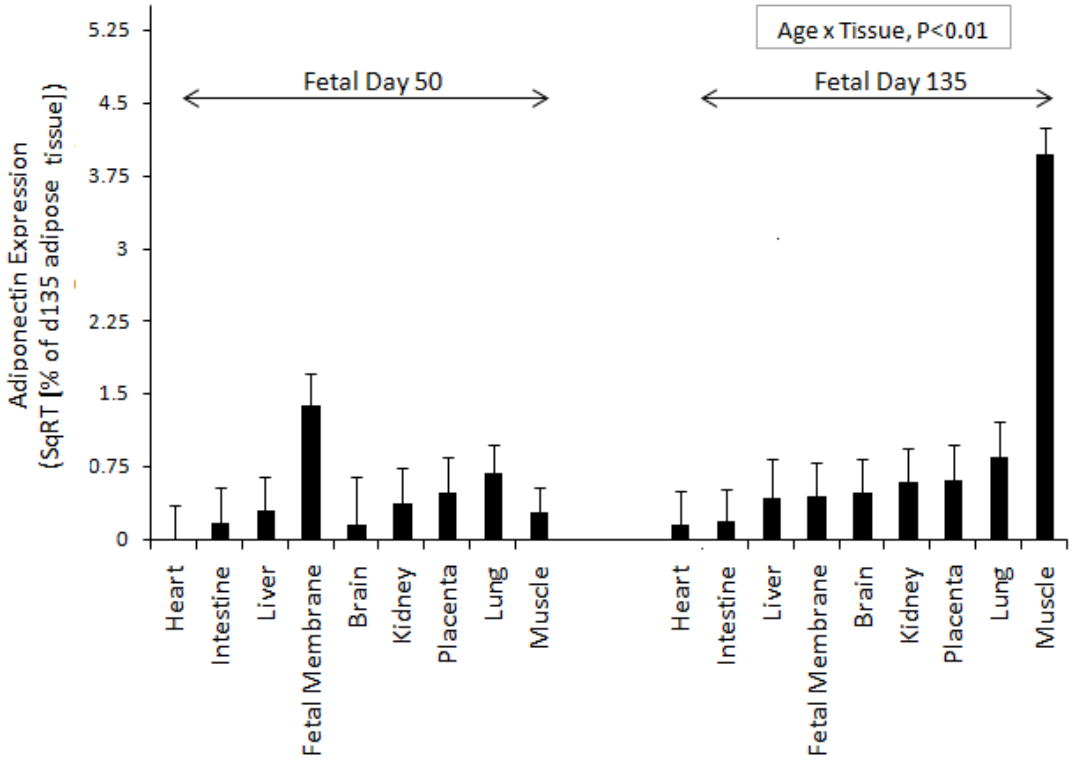

Figure 2: Spatial mRNA of adiponectin in fetal ovine tissues

Tissues were obtained at fetal day 50 or fetal day 135. Adiponectin mRNA relative to 18s rRNA was determined by quantitative real-time RT-PCR and expressed as the square root of the percent of the mean mRNA levels in day 135 perirenal adipose tissue. Each bar represents the mean ± SE of 2-4 fetuses. The significant effect of the interaction between age and tissue is reported.

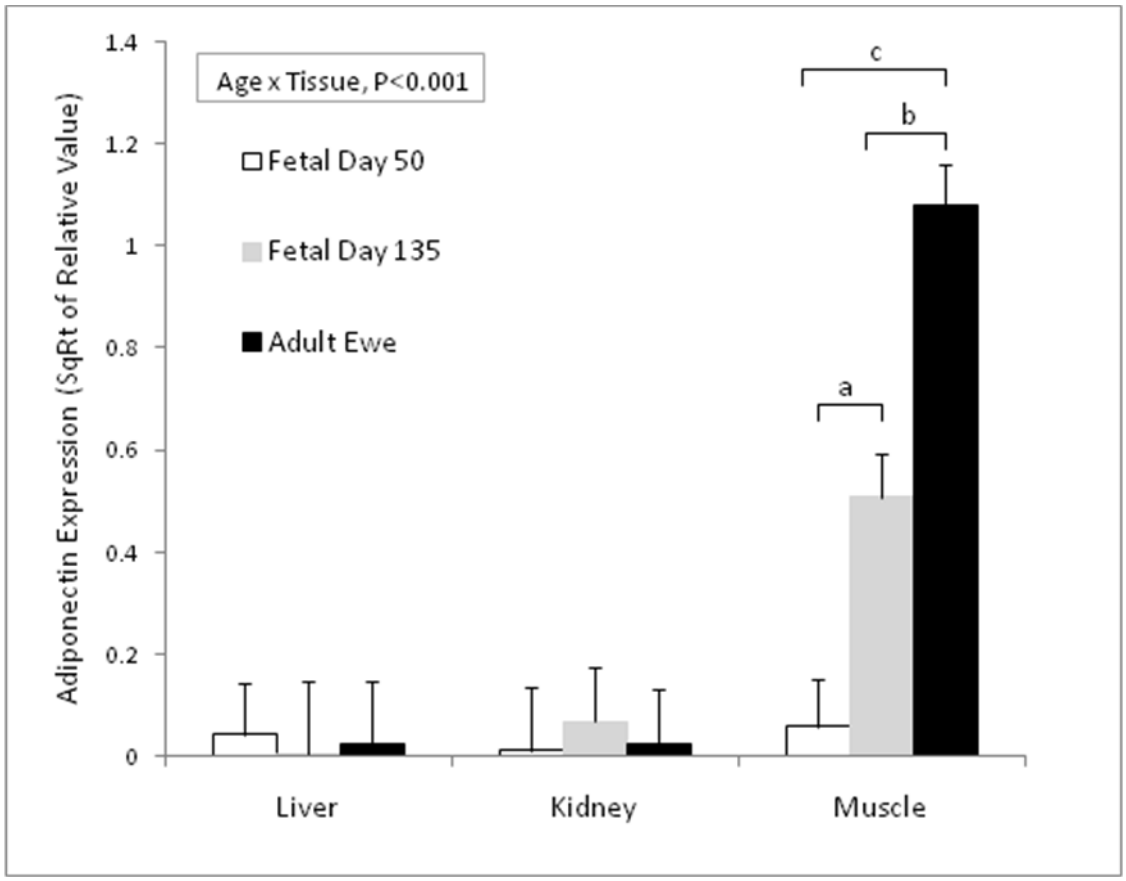

Figure 3: Effect of development on adiponectin mRNA in ovine liver, kidney, and muscle.

Adiponectin mRNA was measured relative to 18s rRNA in tissues obtained during fetal life (day 50 and day 135) and in adult ewes. Adiponectin mRNA was measured by quantitative real-time RT-PCR and reported as the square root of mRNA abundance. Each bar represents the mean ± SE. The analysis of covariance is based on samples of 2-4 animals in each category. The significant effect of the interaction between age and tissue is reported.

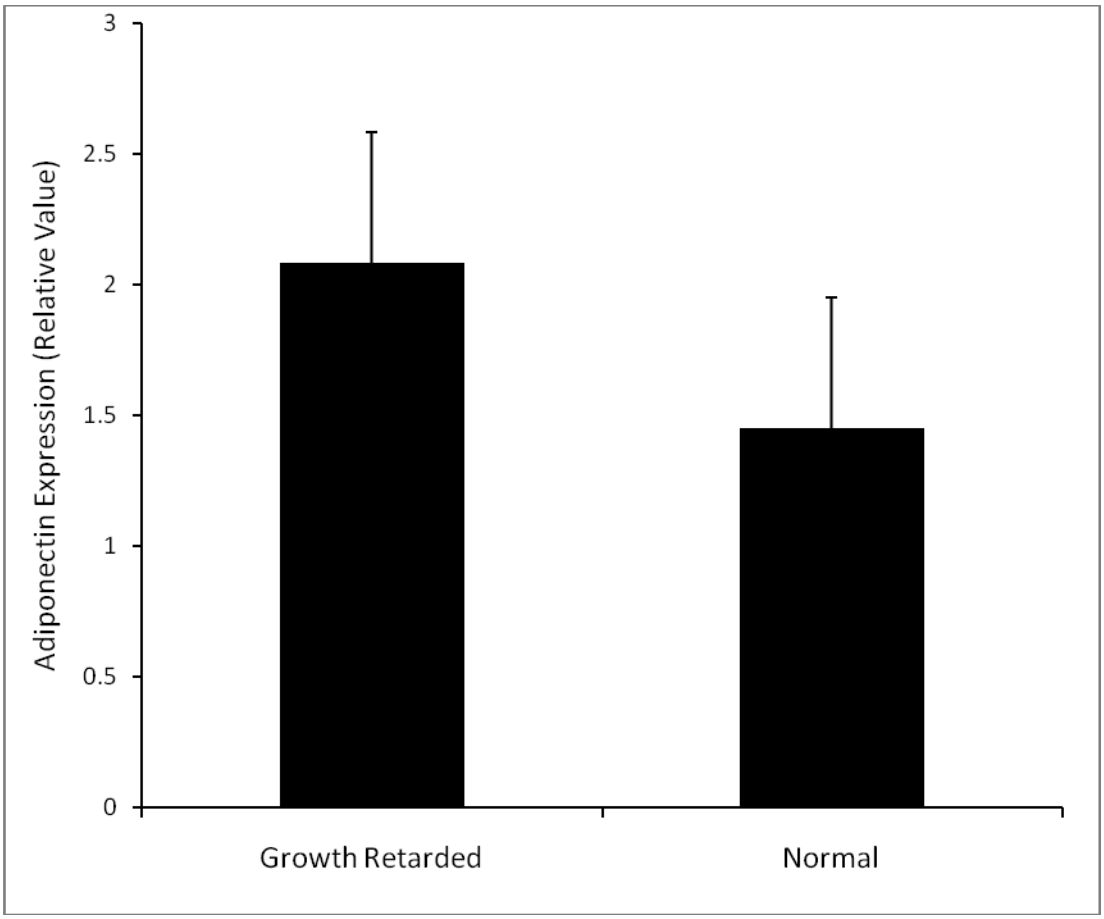

Figure 4: Effect of fetal body size on adiponectin mRNA in ovine perirenal adipose tissue.

Fetuses were obtained at day 135 of fetal life and classified as growth retarded if weighing less than 2.9 kg, and normal if weighing more than 3.8 kg. Adiponectin mRNA was measured relative to 18s rRNA by quantitative real-time RT-PCR. Each bar represents the mean ± SE of 5-6 animals.

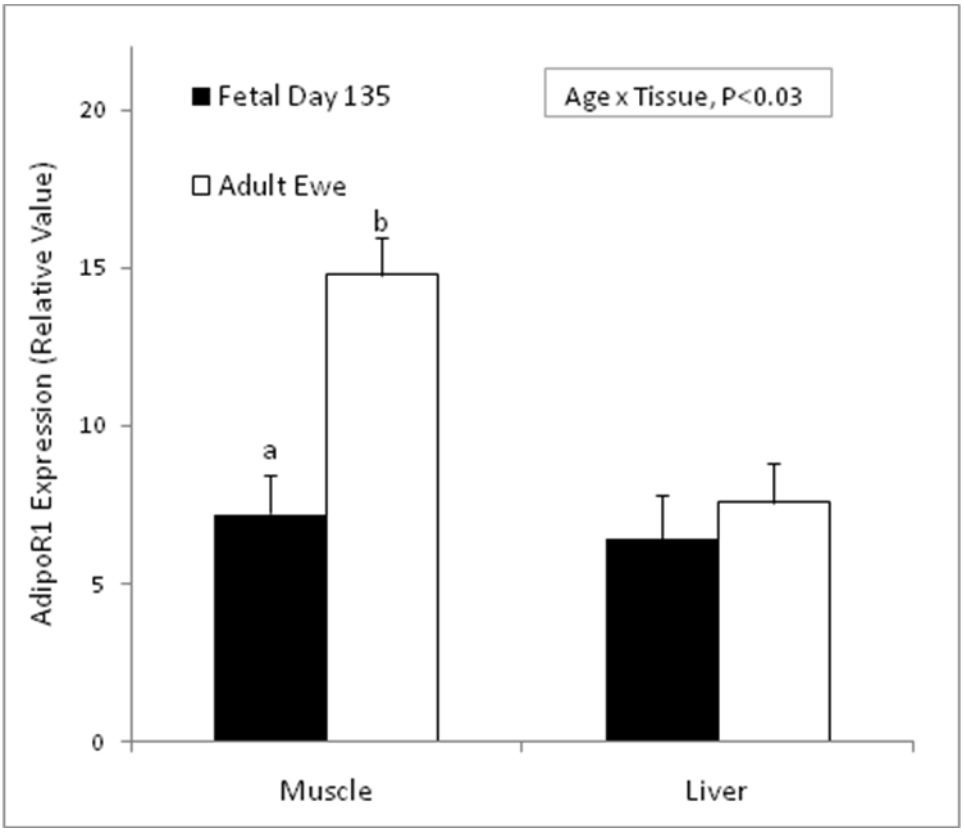

Figure 5: Effect of development on AdipoR1 mRNA in ovine tissues.

Tissues were obtained at fetal day 135 or from adult ewes. AdipoR1 mRNA was measured relative to 18s rRNA by quantitative real-time RT-PCR. Each bar represents the mean ± SE of 6 animals. The significant effect of the interaction between age and tissue is reported. For muscle, bars with different letters differ at $P<0.005$.

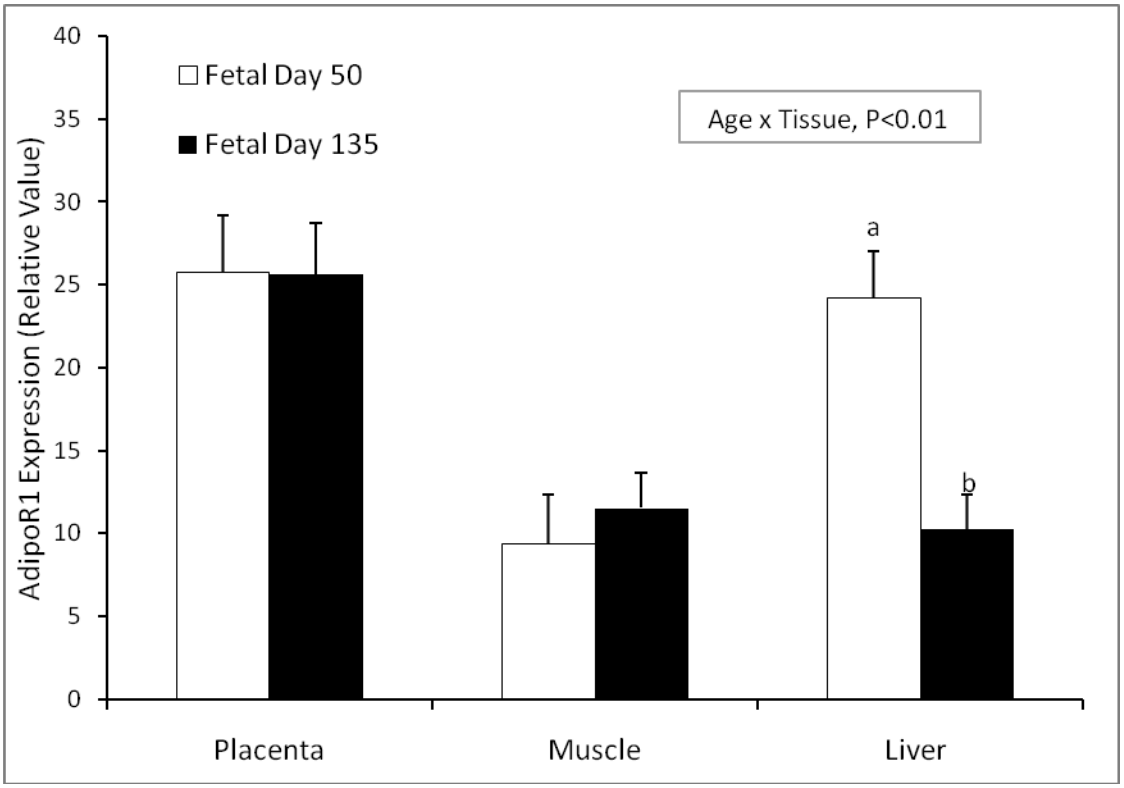

Figure 6: Effect of fetal development on AdipoR1 mRNA in ovine tissues. Tissues were obtained at fetal day 50 or fetal day 135. AdipoR1 mRNA was measured relative to 18s rRNA by quantitative real-time RT-PCR. Each bar represents the mean ± SE of 5-12 animals. The significant effect of the interaction between age and tissue is reported. For liver, bars with different letters differ at $P<0.0001$.

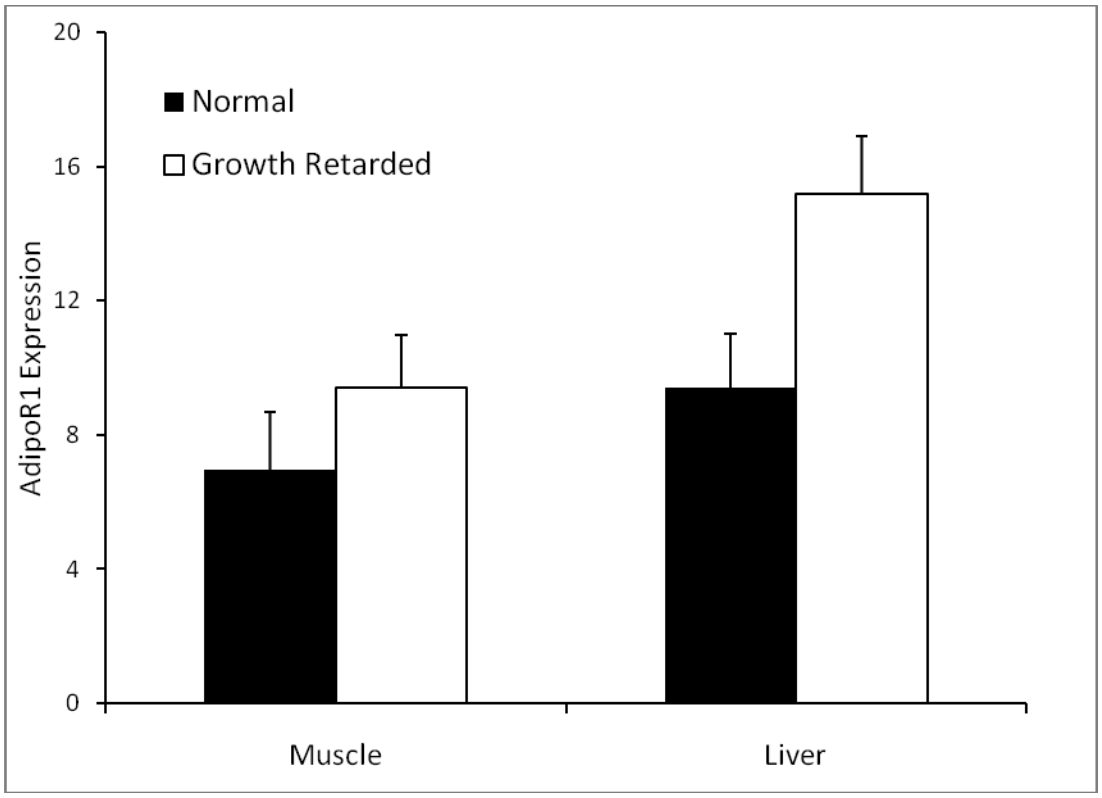

Figure 7: Effect of fetal body size on AdipoR1 mRNA in ovine tissues. Fetuses were obtained at day 135 of fetal life and classified as growth retarded if weighing less than 2.9 kg and normal if weighing more than 3.8 kg. AdipoR1 mRNA was measured relative to 18s rRNA by quantitative real-time RT-PCR. Each bar represents the mean ± SE of 5-6 animals.

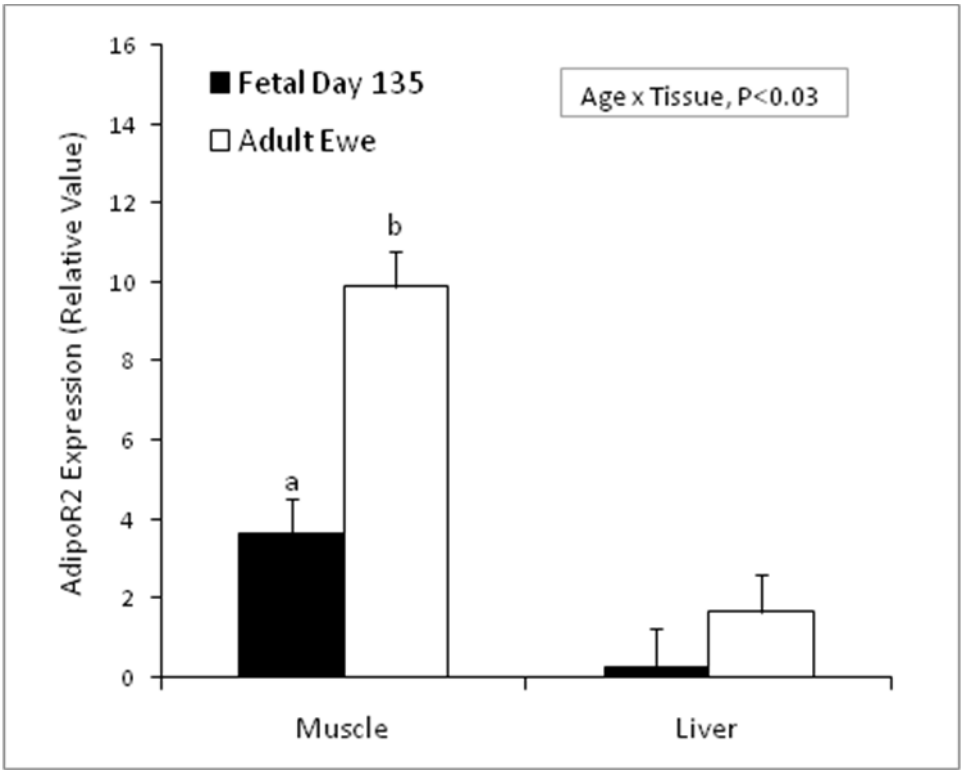

Figure 8: Effect of development on AdipoR2 mRNA in ovine tissues.

Tissues were obtained at fetal day 135 or from adult ewes. AdipoR2 mRNA abundance was measured relative to 18s rRNA by quantitative real-time RT-PCR. Each bar represents the mean ± SE of 6 animals. The significant effect of the interaction between age and tissue is reported. For muscle, bars with different letters differ at $P<0.01$.

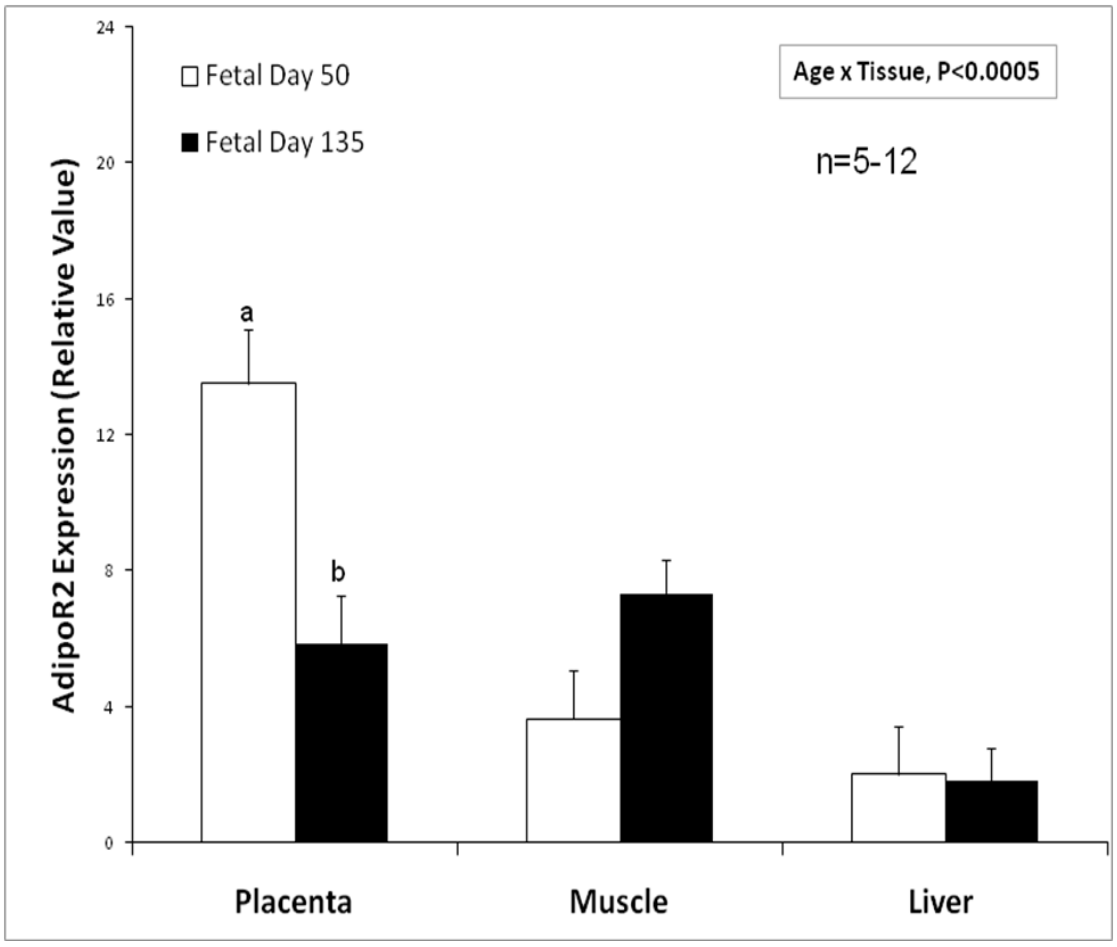

Figure 9**:** Effect of fetal development on AdipoR2 mRNA in ovine tissues. Tissues were obtained at fetal day 50 or fetal day 135. AdipoR2 mRNA was measured relative to 18s rRNA by quantitative real-time RT-PCR. Each bar represents the mean ± SE of 5-12 animals. The significant effect of the interaction between age and tissue is reported. For placenta, bars with different letters differ at $P<0.05$.

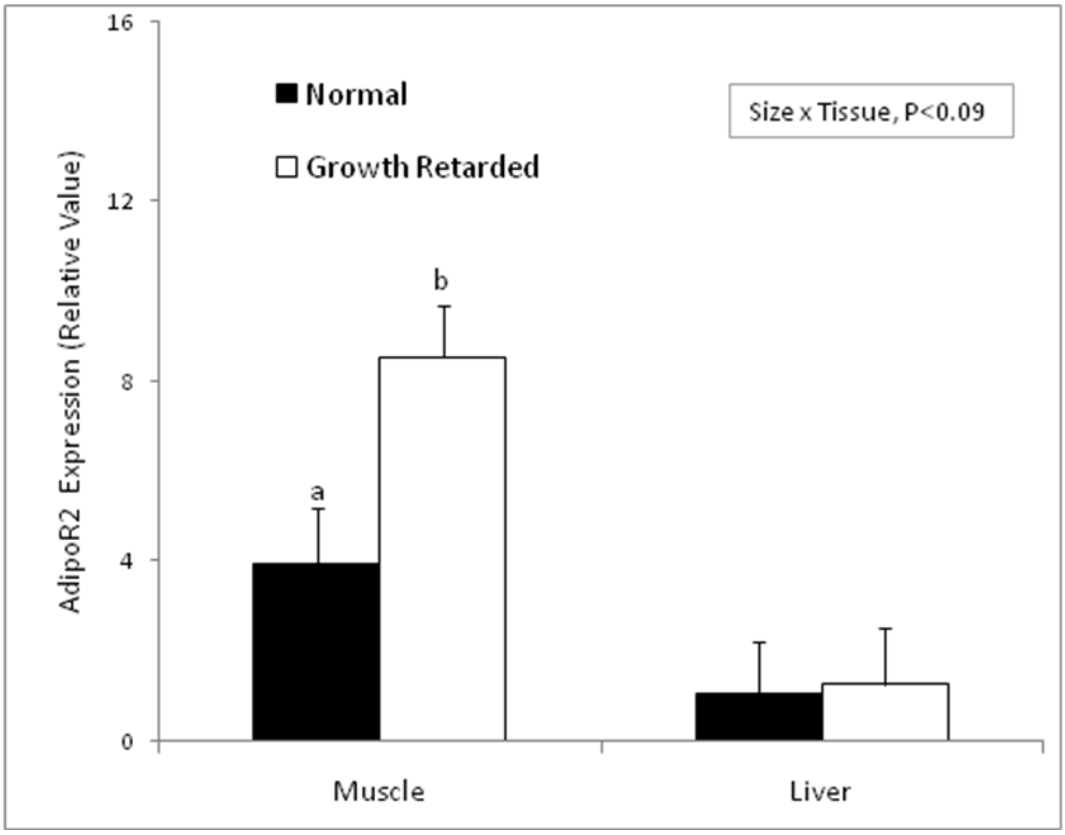

Figure 10: Effect of fetal body size on AdipoR2 mRNA in ovine tissues. Fetuses were obtained at day 135 of fetal life and classified as growth retarded if weighing less than 2.9 kg and normal if weighing more than 3.8 kg. AdipoR2 mRNA was measured relative to 18s rRNA by quantitative real-time RT-PCR. Each bar represents the mean ± SE of 5-6 animals. The tendency of the interaction between size and tissue is reported. For muscle, bars with different letters tend to differ at $P<0.10$.

AdipoR2 mRNA Abundance

AdipoR2 mRNA abundance increased between day 135 of fetal life and adulthood, particularly in muscle (Figure 8, Age x Tissue, $P<0.03$). The increase in muscle mRNA levels was ~3 fold from fetal day 135 to adulthood ($P<0.01$).

The profile of mRNA abundance of AdipoR2 during fetal life differed among tissues (Figure 9, Age x Tissues, $P<0.0005$). Specifically, mRNA levels dropped in placenta between day 50 and day 135 ($P<0.05$). Levels of mRNA in muscle and liver did not change.

Fetal size affected AdipoR2 mRNA abundance in muscle and liver (Figure 10, Tissue x Size, $P<0.09$). This reflected a tendency for increased mRNA levels in skeletal muscle of growth-retarded fetuses ($P<0.10$).

CHAPTER 5
DISCUSSION

Although numerous characterization studies of adiponectin have been performed in humans, little is known about the spatial and temporal regulation of adiponectin and its receptors in sheep during fetal life. One goal of our study was to characterize the mRNA abundance in sheep fetus. In human fetal life, adiponectin mRNA is expressed in tissues of mesodermic origin starting at week 14 (or d98), yet declines with advancing gestational age (Corbetta et al. 2005). Also, in human fetal life, adiponectin is not detected in placenta, but is expressed in various fetal fat tissues (Corbetta et al. 2005).

We found a similar spatial pattern in fetal sheep, with the highest levels of mRNA in the perirenal fat, and a smaller amount detected in the muscle. Interestingly, the level of mRNA in perirenal fat decreases with advancing age, while in muscle, it increases. This temporal pattern of mRNA abundance in fat is expected, since fetal sheep are only 1.8% fat by body weight (Ehrhardt et al. 2002), compared to adult ewes, which have a substantially higher percentage of body fat, depending on feed, breed, etc. The presence of intramuscular fat in the skeletal muscle may explain the detection of adiponectin mRNA in muscle (Vierck et al. 1996).

No adiponectin mRNA abundance was detected in fetal ovine heart, intestine, liver, fetal membrane, brain, lung, or placenta. In the adult chicken, however, the spatial mRNA abundance pattern is quite different than that in sheep, with the exception that in both species, a signal is detected in muscle (Maddineni et al. 2005). However, the remaining tissues (liver, anterior pituitary, diencephalon, and kidney) that exhibit adiponectin expression in chickens are not seen in sheep or humans (Maddineni et al. 2005).

An interesting question to consider is whether or not adiponectin mRNA levels are positively correlated to plasma circulating levels of the protein hormone in fetal sheep life. Previous studies have shown that when adiponectin mRNA abundance is quantified in human adult subjects with varying grades of obesity and insulin sensitivity, that there is a positive correlation between protein circulation and mRNA abundance in adipose tissue (Garaulet et al. 2008). However, there is little evidence that this relationship exists in human or sheep fetal life.

We know the ratio of HMW to total adiponectin is the same in male and female adipocytes in both humans and mice (Xu 2005). Yet, the circulating amount of HMW adiponectin is much higher in females than in males in both species (Xu 2005). This disparity exists because males have higher levels of circulating testosterone, which inhibits the secretion of adiponectin into circulation (Xu 2005). In light of this information in adult mice and humans, it would be interesting to know whether this same phenomenon exists in fetal sheep circulation. Comparing concentrations of molecular forms in fetal and adult sheep in circulation would further help to determine if there exist temporal disparities in circulation.

Our study has shown that IUGR imposed by a physiological model of up to 5 multiple births has no effect on adiponectin or AdipoR1 mRNA abundance in fetal sheep, though a tendency does exist for the mRNA levels of AdipoR2 to be higher in growth retarded muscle. These results, however, are not in line with a human fetal study that shows that adiponectin fetal blood concentration is positively correlated with fetal weight (Sivan et al. 2003). Our results show that there is virtually no difference in adiponectin mRNA levels in perirenal fat from IUGR vs. normal-sized fetuses. Our finding has implications for postnatal sheep life. In humans and rodent models, there is evidence that IUGR or

placental restriction (PR) may lead to obesity in postnatal life (Ross and Beall 2005). Several studies have shown that human fetuses born small for gestational age (SGA) have significantly lower levels of adiponectin compared to their appropriate-for gestational age (AGA) counterparts (Kamoda et al. 2004). This, in turn, may contribute to insulin resistance and metabolic syndrome in postnatal life (Kamoda et al. 2004).

More recent evidence, however, demonstrates that SGA human fetuses secrete a higher amount of HMW adiponectin than their AGA counterparts in an attempt to sensitize themselves to insulin (Ibanez 2008). In any case, adiponectin levels have been shown to be lower in IUGR children that demonstrated catch-up growth compared to IUGR children who remained small throughout childhood (Cianfarani et al. 2004). This implies that infants who exhibit low levels of adiponectin are particularly prone to obesity and insulin resistance in later life. This may occur because adverse uterine environments may permanently damage fetal metabolic pathways, though the mechanisms through which this occurs is not widely known (Cianfarani et al. 2004).

Few studies in sheep have demonstrated that placental restriction has no effect on adiponectin expression (Duffield et al. 2008). There has also been some preliminary work regarding adiponectin mRNA abundance in postnatal lambs, which has shown that an increase of lipogenic enzymes and nuclear receptors, such as PPAR-γ, up-regulates lipogenic hormones such as adiponectin when maternal nutrition is enhanced (Muhlhauser 2007). However, no known study has been performed to establish a connection between the effect of IUGR on receptor mRNA levels in sheep.

Since little is known about the effect of IUGR in adiponectin mRNA levels in postnatal sheep, it would be of interest to us to see if IUGR-subjected

sheep fetuses have reduced insulin sensitivity after birth, despite their similarity in adiponectin mRNA abundance level in adipose tissue compared with normal-sized fetuses in fetal life. It would also be of interest to see if IUGR lambs demonstrate accelerated growth after birth, deemed the "catch-up" period, and how mRNA abundance differs in lambs that display this phenomenon vs. AGA lambs. Since sheep and humans are virtually the same in terms of hypothalamic development and lipid deposition after intrauterine under-nutrition, we would expect lambs to exhibit the same effects as humans (Boyle et al. 1998).

Another goal of the study was to elucidate the spatial mRNA abundance pattern of AdipoR1 and AdipoR2 in sheep. In humans and mice, R1 mRNA abundance is most abundant in adult skeletal muscle, while R2 is most abundant in adult liver (Zhang 2005). However, in sheep, both receptors are most abundantly expressed in the placenta. AdipoR1 shows a higher level of mRNA abundance in the liver than in muscle in fetal life, while AdipoR2 was more abundant in muscle than in liver in fetal life. In addition, this study not only provides evidence that IUGR (and thereby, fetal size) has no effect on level of adiponectin mRNA abundance, but also adds to this line of knowledge by showing that it has no bearing on the mRNA abundance level AdipoR1 or AdipoR2.

In summary, our studies show that in fetal sheep, adiponectin mRNA is predominantly expressed in fat tissue with a small amount in muscle due to its intramuscular fat content. AdipoR1 and AdipoR2 mRNA are both expressed in muscle, liver, and placenta, yet a functional role for these sources has yet to be clearly defined. Fetal sheep are an appropriate model for these studies, since their endocrine and metabolic systems are similar to the human metabolic system (Ehrhardt et al. 2002).

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

In humans, adiponectin mRNA expression is exclusively in the adipose tissue (Kern 2003). Also, it is well-documented that there is a significant positive correlation between adiponectin mRNA expression in adipose and circulating plasma levels of the protein hormone (Garaulet et al. 2008). This study has shown that adiponectin mRNA is expressed exclusively in adipose tissue in fetal sheep. However, our study did not examine whether or not this mRNA abundance positively correlates to circulating plasma amount of adiponectin. Therefore, the next logical step would involve performing Western blots on various maternal and fetal plasma samples to establish if a relationship exists.

Such subsequent experiments would help us pose further questions. For example, if there is a negative correlation between mRNA and plasma expression, perhaps a thiol-mediated retention, which is present in humans, is also present in fetal sheep adipocytes (Anelli 2003). Since this retention is thought to ensure proper assembly of the HMW form, this observation may lead to further analysis of concentrations of the three different molecular forms in fetal sheep plasma (Anelli 2003). Of particular interest to our research group is whether IUGR (small) fetuses contain a different ratio of HMW to total adiponectin compared to normal-sized fetuses. The same ratio of the respective maternal plasma samples of these small and normal- sized fetuses would also need to be characterized to elucidate a correlation between their concentrations and their offspring. Examining maternal concentrations would

also be helpful in determining whether or not respective isoforms decrease in the ewes and increase in fetuses, or vice versa.

Yet another interesting future direction of this work may lie in analyzing insulin sensitivity of IUGR versus normal-sized fetuses. Since our study has shown that there is no difference in adiponectin mRNA abundance between IUGR and normal-sized fetuses, it would be useful to know whether this observation has a direct relationship to insulin sensitivity. It is also of use to know if insulin sensitivity declines or increases with advancing gestational age in these fetuses. Since there is evidence in humans that maternal insulin-sensitivity declines as gestation progresses, a similar decline in ovine maternal sensitivity and increase in fetal sheep sensitivity with advancing gestation will allow us to establish a key similarity between sheep and humans. Once again, if a high HMW to total adiponectin ratio is found in fetal sheep plasma, this may also help us form the conclusion that the HMW has a more insulin-sensitizing effect than the other two molecular forms, as is the case in humans (Wang 2008).

A serious limitation of our study was not having the ability to characterize adiponectin and receptor mRNA levels in nutritionally-modified fetal sheep. It is well-documented that mice which are fed a high-fat diet exhibit weight gain, and elevated plasma glucose levels associated with hyperinsulinemia (Barnea et al. 2006). These mice also show a decrease in adiponectin mRNA levels in adipose tissues (Barnea et al. 2006). However, their serum adiponectin levels remain unchanged when compared with control groups (Barnea et al. 2006). Abundance of hepatic mRNA for AdipoR1 and AdipoR2 is reduced by 15% and 25%, respectively, in mice fed the HF diet. In contrast, mRNA abundance for AdipoR1 and AdipoR2 increased by 25% and

30%, respectively, in muscle tissue (Barnea et al. 2006). These results imply that the HF diet led to decreased insulin sensitivity accompanied by impaired activity of adiponectin-related enzymes in skeletal muscles but not in the liver (Barnea et. al 2006).

The prospect of determining whether or not receptor mRNA levels differs between muscle and liver obtained from a fetus whose mother was fed a high-plane of nutrition throughout her pregnancy versus a low plane of nutrition fetus is useful in determining if maternal nutrition modifies levels of mRNA for adiponectin receptor in sheep. There is evidence that ewes fed a diet of excess energy requirements, in turn, over-express lipogenic enzymes such as PPAR-γ (Muhlhausler 2006). However, a direct relationship between adiponectin and receptor mRNA concentration in sheep fetuses with overfed mothers has not yet been ascertained.

BIBLIOGRAPHY

Andersen, K. K., et al. "Gender Differences of Oligomers and Total Adiponectin during Puberty: A Cross-Sectional Study of 859 Danish School Children." *The Journal of clinical endocrinology and metabolism* 92.5 (2007): 1857-62.

Anelli, T., et al. "Thiol-Mediated Protein Retention in the Endoplasmic Reticulum: The Role of ERp44." *The EMBO journal* 22.19 (2003): 5015-22.

Barnea, M., et al. "A High-Fat Diet has a Tissue-Specific Effect on Adiponectin and Related Enzyme Expression." *Obesity (Silver Spring, Md.)* 14.12 (2006): 2145-53.

Barry, J. S., and R. V. Anthony. "The Pregnant Sheep as a Model for Human Pregnancy." *Theriogenology* 69.1 (2008): 55-67.

Basu, S., et al. "In Utero Gender Dimorphism of Adiponectin Reflects Insulin Sensitivity and Adiposity of the Fetus." *Obesity (Silver Spring, Md.)* (2009).

Bjursell, M., et al. "Opposing Effects of Adiponectin Receptors 1 and 2 on Energy Metabolism." *Diabetes* 56.3 (2007): 583-93.

Bobbert, T., et al. "Changes of Adiponectin Oligomer Composition by Moderate Weight Reduction." *Diabetes* 54.9 (2005): 2712-9.

Caminos, J. E., et al. "Expression and Regulation of Adiponectin and Receptor in Human and Rat Placenta." *The Journal of clinical endocrinology and metabolism* 90.7 (2005): 4276-86.

Chen, H., et al. "Adiponectin Stimulates Production of Nitric Oxide in Vascular Endothelial Cells." *The Journal of biological chemistry* 278.45 (2003): 45021-6.

Cianfarani, S., et al. "Adiponectin Levels are Reduced in Children Born Small for Gestational Age and are Inversely Related to Postnatal Catch-Up Growth." *The Journal of clinical endocrinology and metabolism* 89.3 (2004): 1346-51.

Combs, T. P., et al. "Induction of Adipocyte Complement-Related Protein of 30 Kilodaltons by PPARgamma Agonists: A Potential Mechanism of Insulin Sensitization." *Endocrinology* 143.3 (2002): 998-1007.

Corbetta, S., et al. "Adiponectin Expression in Human Fetal Tissues during Mid- and Late Gestation." *The Journal of clinical endocrinology and metabolism* 90.4 (2005): 2397-402.

Crimmins, N. A., and L. J. Martin. "Polymorphisms in Adiponectin Receptor Genes ADIPOR1 and ADIPOR2 and Insulin Resistance." *Obesity reviews : an official journal of the International Association for the Study of Obesity* 8.5 (2007): 419-23.

Ebinuma, H., et al. "Improved ELISA for Selective Measurement of Adiponectin Multimers and Identification of Adiponectin in Human Cerebrospinal Fluid." *Clinical chemistry* 53.8 (2007): 1541-4.

Ehrhardt, R. A., A. W. Bell, and Y. R. Boisclair. "Spatial and Developmental Regulation of Leptin in Fetal Sheep." *American journal of physiology.Regulatory, integrative and comparative physiology* 282.6 (2002): R1628-35.

Fu, Y., et al. "Adiponectin Promotes Adipocyte Differentiation, Insulin Sensitivity, and Lipid Accumulation." *Journal of lipid research* 46.7 (2005): 1369-79.

Garaulet, M., et al. "Adiponectin, the Controversial Hormone." *Public health nutrition* 10.10A (2007): 1145-50.

Gonzalez-Sanchez, J. L., et al. "An SNP in the Adiponectin Gene is Associated with Decreased Serum Adiponectin Levels and Risk for Impaired Glucose Tolerance." *Obesity research* 13.5 (2005): 807-12.

Greenwood, P. L., et al. "Effects of Birth Weight and Postnatal Nutrition on Neonatal Sheep: I. Body Growth and Composition, and some Aspects of Energetic Efficiency." *Journal of animal science* 76.9 (1998): 2354-67.

Hada, Y., et al. "Selective Purification and Characterization of Adiponectin Multimer Species from Human Plasma." *Biochemical and biophysical research communications* 356.2 (2007): 487-93.

Hopkins, T. A., et al. "Adiponectin Actions in the Cardiovascular System." *Cardiovascular research* 74.1 (2007): 11-8.

Hug, C., et al. "T-Cadherin is a Receptor for Hexameric and High-Molecular-Weight Forms of Acrp30/adiponectin." *Proceedings of the National Academy of Sciences of the United States of America* 101.28 (2004): 10308-13.

Ibanez, L., et al. "Abdominal Fat Partitioning and High-Molecular-Weight Adiponectin in Short Children Born Small for Gestational Age." *The Journal of clinical endocrinology and metabolism* 94.3 (2009): 1049-52.

Kadowaki, T., and T. Yamauchi. "Adiponectin and Adiponectin Receptors." *Endocrine reviews* 26.3 (2005): 439-51.

Kamoda, T., et al. "Serum Adiponectin Concentrations in Newborn Infants in Early Postnatal Life." *Pediatric research* 56.5 (2004): 690-3.

Kern, P. A., et al. "Adiponectin Expression from Human Adipose Tissue: Relation to Obesity, Insulin Resistance, and Tumor Necrosis Factor-Alpha Expression." *Diabetes* 52.7 (2003): 1779-85.

Kobayashi, H., et al. "Selective Suppression of Endothelial Cell Apoptosis by the High Molecular Weight Form of Adiponectin." *Circulation research* 94.4 (2004): e27-31.

Kubota, N., et al. "Disruption of Adiponectin Causes Insulin Resistance and Neointimal Formation." *The Journal of biological chemistry* 277.29 (2002): 25863-6.

Kusminski, C. M., et al. "Adiponectin Complexes in Human Cerebrospinal Fluid: Distinct Complex Distribution from Serum." *Diabetologia* 50.3 (2007): 634-42.

Maddineni, S., et al. "Adiponectin Gene is Expressed in Multiple Tissues in the Chicken: Food Deprivation Influences Adiponectin Messenger Ribonucleic Acid Expression." *Endocrinology* 146.10 (2005): 4250-6.

Maeda, N., et al. "PPARgamma Ligands Increase Expression and Plasma Concentrations of Adiponectin, an Adipose-Derived Protein." *Diabetes* 50.9 (2001): 2094-9.

Menzaghi, C., V. Trischitta, and A. Doria. "Genetic Influences of Adiponectin on Insulin Resistance, Type 2 Diabetes, and Cardiovascular Disease." *Diabetes* 56.5 (2007): 1198-209.

Merl, V., et al. "Serum Adiponectin Concentrations during a 72-Hour Fast in Over- and Normal-Weight Humans." *International journal of obesity (2005)* 29.8 (2005): 998-1001.

Metais, C., et al. "Adiponectin Receptors: Expression in Zucker Diabetic Rats and Effects of Fenofibrate and Metformin." *Metabolism: clinical and experimental* 57.7 (2008): 946-53.

Muhlhausler, B. S., J. A. Duffield, and I. C. McMillen. "Increased Maternal Nutrition Increases Leptin Expression in Perirenal and Subcutaneous Adipose Tissue in the Postnatal Lamb." *Endocrinology* 148.12 (2007): 6157-63.

Nakano, Y., et al. "A Novel Enzyme-Linked Immunosorbent Assay Specific for High-Molecular-Weight Adiponectin." *Journal of lipid research* 47.7 (2006): 1572-82.

Niemann, B., R. E. Silber, and S. Rohrbach. "Age-Specific Effects of Short- and Long-Term Caloric Restriction on the Expression of Adiponectin and Adiponectin Receptors: Influence of Intensity of Food Restriction." *Experimental gerontology* 43.7 (2008): 706-13.

Ocon-Grove, O. M., et al. "Adiponectin and its Receptors are Expressed in the Chicken Testis: Influence of Sexual Maturation on Testicular ADIPOR1 and ADIPOR2 mRNA Abundance." *Reproduction (Cambridge, England)* 136.5 (2008): 627-38.

Pajvani, U. B., et al. "Complex Distribution, Not Absolute Amount of Adiponectin, Correlates with Thiazolidinedione-Mediated Improvement in Insulin Sensitivity." *The Journal of biological chemistry* 279.13 (2004): 12152-62.

Peake, P. W., et al. "Glycosylation of Human Adiponectin Affects its Conformation and Stability." *Journal of Molecular Endocrinology* 39.1 (2007): 45-52.

Qiang, L., H. Wang, and S. R. Farmer. "Adiponectin Secretion is Regulated by SIRT1 and the Endoplasmic Reticulum Oxidoreductase Ero1-L Alpha." *Molecular and cellular biology* 27.13 (2007): 4698-707.

Ramachandran, R., O. M. Ocon-Grove, and S. L. Metzger. "Molecular Cloning and Tissue Expression of Chicken AdipoR1 and AdipoR2 Complementary Deoxyribonucleic Acids." *Domestic animal endocrinology* 33.1 (2007): 19-31.

Richards, A. A., et al. "Adiponectin Multimerization is Dependent on Conserved Lysines in the Collagenous Domain: Evidence for Regulation of Multimerization by Alterations in Posttranslational Modifications." *Molecular endocrinology (Baltimore, Md.)* 20.7 (2006): 1673-87.

Ross, M. G., and M. H. Beall. "Adult Sequelae of Intrauterine Growth Restriction." *Seminars in perinatology* 32.3 (2008): 213-8.

Scherer, P. E., et al. "A Novel Serum Protein Similar to C1q, Produced Exclusively in Adipocytes." *The Journal of biological chemistry* 270.45 (1995): 26746-9.

Schraw, T., et al. "Plasma Adiponectin Complexes have Distinct Biochemical Characteristics." *Endocrinology* 149.5 (2008): 2270-82.

Semple, R. K., et al. "Elevated Plasma Adiponectin in Humans with Genetically Defective Insulin Receptors." *The Journal of clinical endocrinology and metabolism* 91.8 (2006): 3219-23.

Sivan, E., et al. "Adiponectin in Human Cord Blood: Relation to Fetal Birth Weight and Gender." *The Journal of clinical endocrinology and metabolism* 88.12 (2003): 5656-60.

Trujillo, M. E., and P. E. Scherer. "Adipose Tissue-Derived Factors: Impact on Health and Disease." *Endocrine reviews* 27.7 (2006): 762-78.

Tsao, T. S., et al. "Role of Disulfide Bonds in Acrp30/adiponectin Structure and Signaling Specificity. Different Oligomers Activate Different Signal Transduction Pathways." *The Journal of biological chemistry* 278.50 (2003): 50810-7.

Vierck, J. L., J. P. McNamara, and M. V. Dodson. "Proliferation and Differentiation of Progeny of Ovine Unilocular Fat Cells (Adipofibroblasts)." *In vitro cellular & developmental biology.Animal* 32.9 (1996): 564-72.

Waki, H., et al. "Impaired Multimerization of Human Adiponectin Mutants Associated with Diabetes. Molecular Structure and Multimer Formation of Adiponectin." *The Journal of biological chemistry* 278.41 (2003): 40352-63.

Wang, Y., et al. "Post-Translational Modifications of Adiponectin: Mechanisms and Functional Implications." *The Biochemical journal* 409.3 (2008): 623-33.

Wang, Z. V., et al. "PANIC-ATTAC: A Mouse Model for Inducible and Reversible Beta-Cell Ablation." *Diabetes* 57.8 (2008): 2137-48.

Wang, Z. V., et al. "Secretion of the Adipocyte-Specific Secretory Protein Adiponectin Critically Depends on Thiol-Mediated Protein Retention." *Molecular and cellular biology* 27.10 (2007): 3716-31.

Whitehead, J. P., et al. "Adiponectin--a Key Adipokine in the Metabolic Syndrome." *Diabetes, obesity & metabolism* 8.3 (2006): 264-80.

Xu, A., et al. "The Fat-Derived Hormone Adiponectin Alleviates Alcoholic and Nonalcoholic Fatty Liver Diseases in Mice." *The Journal of clinical investigation* 112.1 (2003): 91-100.

Zhang, J., et al. "Plasma Adiponectin Concentrations are Independently Predicted by Fat Insulin Sensitivity in Women and by Muscle Insulin Sensitivity in Men." *Diabetes care* 28.3 (2005): 755-6.